Application Note: PI4015.0307

Introduction to the Biological Production of Large Molecule Pharmaceuticals

Exhibit A

Key Words
- Fermentation
- Fermentor
- Fermenter
- Cell Culture
- Biologics
- Bioprocess
- PAT
- Bioremediation
- Proteins
- Enzymes

Introduction

As a result of regulatory oversight, the manufacturing techniques of the pharmaceutical industry have lagged far behind those of other chemical processing industries. In the past, adding new Process Analytical Technology (PAT) for the purpose of process optimization would almost certainly have resulted in the suspension of all manufacturing permits until the new manufacturing system had been fully validated. The resulting delays and risks have been too much for the pharmaceutical industry to assimilate. This is understandable when consideration is given to the time it takes to bring a new product to market (10-15 years) and the compressed patent protection that results from the years of clinical trials that each new product must go through. Recent policy changes at the US Food and Drug Administration (FDA) are rapidly reversing this trend. In spite of the historical regulatory pressures, however, there is one area of pharmaceutical manufacturing that has often made use of extensive online PAT—the biological production area. This is where active ingredients are manufactured using living cells as opposed to the more common methods of traditional chemical synthesis. The complexity and delicacy of these microbiological processes have always required a fair degree of online instrumentation. Process understanding results from the study of the organisms, their nutritional requirements and their metabolic products in small-scale, bench-top reactors using online instrumentation. The scale-up process provides a natural migration of analytical technology from bench scale through pilot scale to full-scale biopharmaceutical manufacturing. The use of PAT for biological production is the topic of this industrial solutions note.

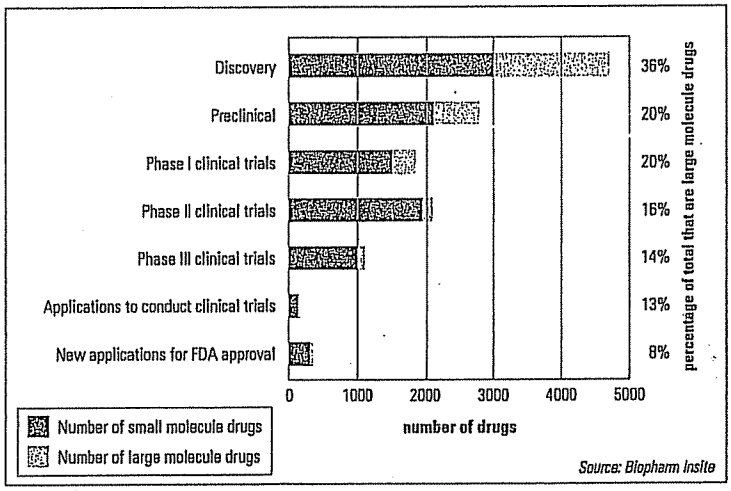

figure 1 – Biopharmaceutical vitality

Biopharmaceutical Vitality

Whereas 95% of pharmaceutical active ingredients are currently based on chemically synthesized small molecules, protein-based large molecule ingredients are becoming increasingly used to provide novel therapeutical solutions. *Figure 1* illustrates the trend that has resulted from the recent advances in the understanding of the human genome. Now, more than a third of all drug candidates emerging from drug discovery are large molecule proteins. The growth of the biological sector will continue to outpace the small molecule sector in the coming decade.

Figures 2 and 3 illustrate how the manufacturing process differs for small molecule and large molecule active pharmaceutical ingredients (variously referred to as "active" or API). Aspirin is chosen to represent a typical small molecule that is assembled by the batch reaction of three chemicals: Carboxyl Methyl Cellulose, Acetic Anhydride, and Salicylic Acid. The aspirin is extracted from the batch reactor along with other products and un-reacted feedstock. The next step is to separate the aspirin from the rest of the material. These processes will provide a consistent quality product as long as the ingredients are always pure and the standard operating procedures are followed to the letter. Similar assumptions are made regarding the secondary manufacturing processes of purification, drying, formulation (the addition of inert exipients) and tableting.

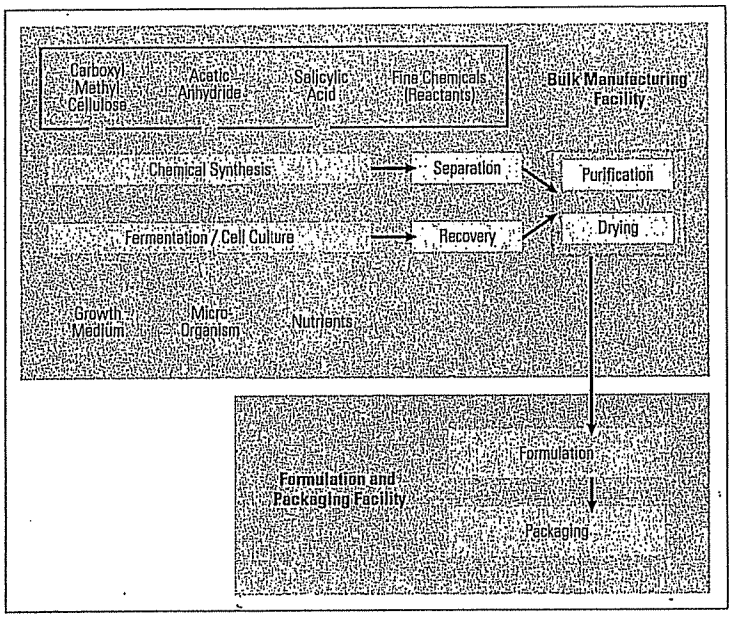

figure 2 – Biopharmaceutical manufacturing flow

Testing for final product quality has traditionally occurred by post-manufacturing laboratory analysis of a small randomly selected batch of tablets. Any substandard parameters of the test batch result in the loss of the entire production batch. The PAT initiative is designed to make substantial improvements to this paradigm. The new approach is, wherever possible, to make informative measurements online in areas where there is the greatest risk of introducing poor quality. If the analytical measurements are based on sound science, the data can be relied upon to improve understanding of the processes, enabling quality to be designed into the modernized production line. The regulating authorities are now providing a "safe harbor" for pharmaceutical manufacturers that can demonstrate such a science-based approach.

Whereas the traditional procedure-based methods of chemical synthesis have been sustainable for small molecule API, that has not been the case for protein products. Manufacturing these large molecules is far more complicated. These proteins are made from a sequence of amino acids, often hundreds of units long, that fold into complicated shapes. More often than not, it is the shape of the molecule that triggers the desired biochemical reaction when the drug is administered. The traditional method of building these complicated molecules is to rely on microorganisms that have evolved over billions of years, to be extremely efficient protein factories. In situations where a cell that makes the target molecule naturally cannot be found, the cell's DNA can be modified by splicing in a gene that codes for the target molecule. The DNA molecule can be thought of as a library of all the software routines that are required to program all the cellular operations. The individual gene is then a subroutine that contains the recipe for the protein and the instructions for ordering its assembly. A gene is said to be "expressed" when the subroutine is operated and the target molecule is manufactured by the cell. One additional complication is that cells often have several genes that regulate each other. The microbiologist often needs to construct a detailed map of the cascading chemical reactions (the metabolic pathways) in order to understand the cell well enough to design a viable manufacturing process. Once the cell has been selected, it is necessary to design a matrix of nutrients (the growth medium) that will first encourage the cells to rapidly multiply, and then finally establish the conditions that will trigger sustainable production of the target molecule. Since live, fragile cells are used in the process, there are more things that can go wrong to affect the outcome than can be expected with traditional chemical synthesis.

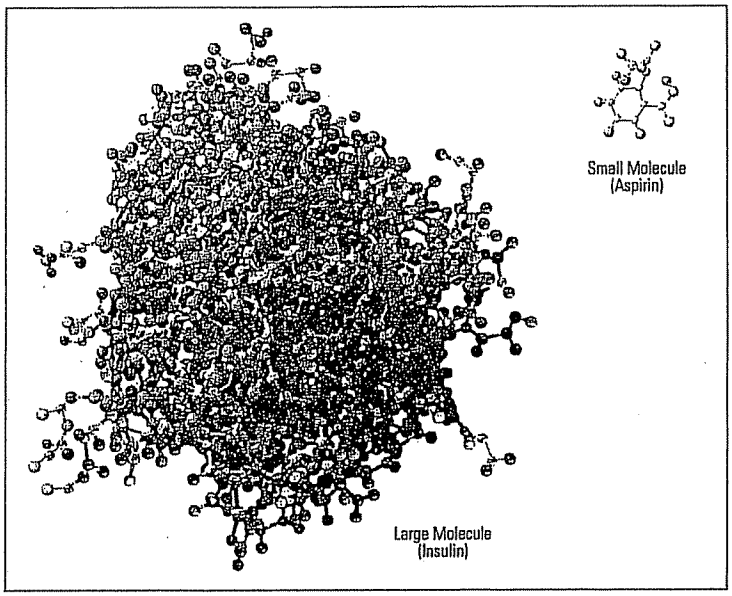

figure 3 – Sample molecules in relative sizes

Microbial Fermentation (fungi, protozoa, bacteria and archaea)

Bacteria and archaea are members of the prokaryotes domain—literally "before the nucleus." These are ancient cells that have existed for billions of years and have been successfully co-opted by microbiologists as the basis of many robust biological manufacturing processes. The archaea are truly ancient bacteria that now mainly occupy only extreme environments, e.g., very salty environments (extreme halophiles) and extreme temperatures (hyperthermophiles). Fungi and animal cells, on the other hand, are eukaryotes. Their domain name means "true kernel;" the kernel being the nucleus of the eukaryotic cell. The nucleus contains the cell's genetic material, with DNA (short for dioxyribonucleic acid) being the most important of these materials. Eukaryotes have their DNA in linear strings that are bound up with proteins to make chromosomes (normally visible only when the cells are dividing—about 3% of the time).

Cellular Respiration

Bacteria, like all other organisms, need energy to live; they obtain this energy by respiration. Most eukaryotes and prokaryotes use aerobic respiration to obtain energy from glucose. The mitochondrion of a cell are frequently referred to as the "power plants" of the cell. This is where the reactions that create energy (in the form of ATP) take place. Mitochondria replicate independently of the cell and therefore contain their own genetic material, a circular strand of DNA that is completely unlike the DNA found in the rest of the cell. Respiration usually involves oxygen which is used to power the breakdown of food molecules to release energy. In some cases, these organisms can utilize a range of alternatives to oxygen for the purpose of respiration. These include elemental sulfur, nitrogen, nitrogen oxides and various organic species. These attributes contribute to the ability of prokaryotes to colonize many of Earth's most hostile environments.

The overall respiration reaction is:

$C_6H_{12}O_6 + 6O_2$ *yields*
$6CO_2 + 6H_2O$ *+ energy (as ATP)*

Protein Production

The Rough Endoplasmic Reticulum (RER) is where the ribosomes are located. In fact, they are called "rough" because they are studded with ribosomes, which are small organelles that synthesize protein. These processing sites translate any messenger RNA (mRNA) in the cell. The first part of the process is to identify the "start" codon. The proteins in the ribosome then join the amino acids together to make the proteins dictated by the sequence on the mRNA. In the final step, a "stop" codon is identified and the chain is broken. The new chain of amino acids is released into the cell.

Process Development

During the early stages of process development, the cells are grown in a shake flask from isolated colonies that are stored

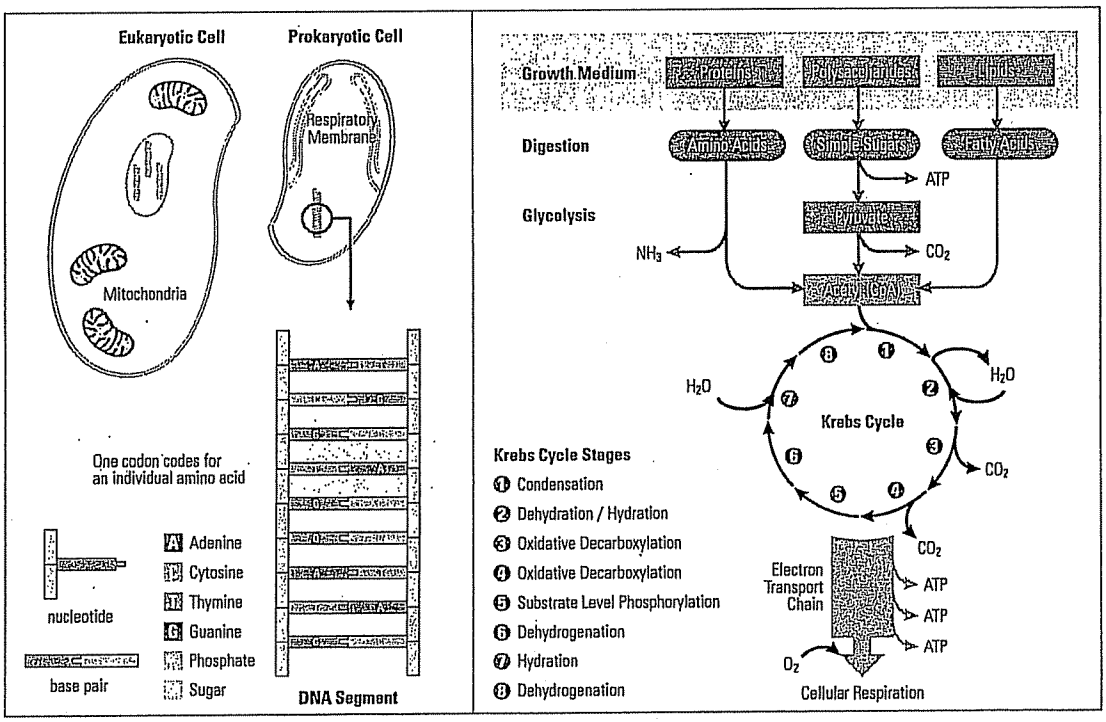

figure 4 – Overview of the key biological processes

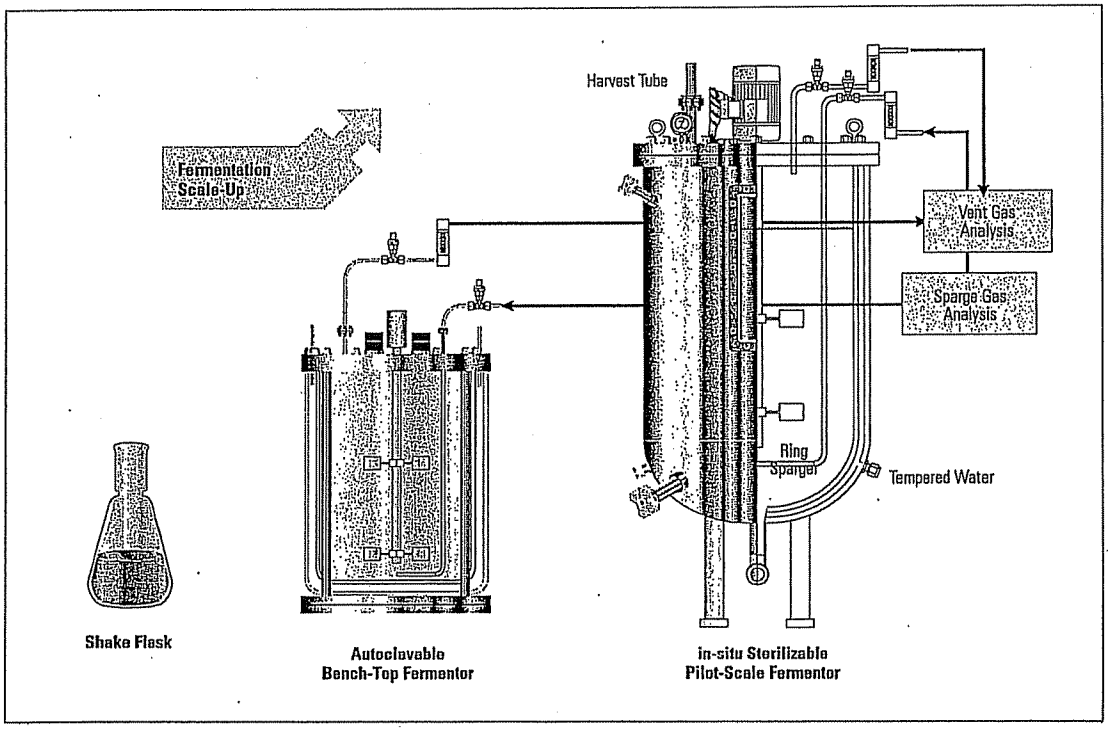

figure 5 – Research and development scale-up

in a -80°C freezer. The flask will contain a sterile broth of nutrients and will be located in an incubator maintained at 37°C. The cells are usually grown overnight and then the culture is used to inoculate a larger 2 to 5-liter bench-top fermentor. A typical development lab will have a large number of these, each fermenting different strains or using different recipes of broth. Each of these reactors will be sparged with air to ensure that the cells get an adequate supply of oxygen. A fully equipped lab will include a mass spectrometer in order to monitor the effluent gases from each fermentor. The gas concentration data are used to calculate real time values for biomass growth and respiration quotient (RQ) and also to monitor various metabolic products that can be detected at parts per million (ppm) concentrations in the off gas. The purpose of using a large number of variations is to determine by trial and error the most potent combination of microbial strain and broth recipe. In some cases, the nutrient mix is changed on-the-fly as the biomass increases. The mass spectrometer (MS) data are used to automate this "fed batch" process.

Once the process has been optimized at the 5-liter scale, the process moves up to 200 liters (typically). This is usually referred to as pilot scale. A fermentor of this size will normally be heavily instrumented with probes to monitor pH, turbidity (fine particulates), dissolved oxygen, dissolved carbon dioxide (important for mammalian cells) and of course temperature. Fermentation is exothermic (heat generating) so cooling water is necessary to remove excess heat. Analysis of the top gas will be used to test for sterility prior to inoculation and then for respiration activity during the growth phase. The mass spectrometer will also be used to check for metabolites that might indicate poor aeration, nutrient imbalance or toxic buildup. The final step is to go to full scale commercial production. This could be as much as 50,000 to 100,000 liters per batch.

Downstream Processing

Once the batch process has run its course, the fermentor is chilled in order to slow down metabolic activity; this promotes stability prior to product recovery. The exact design of the recovery system will depend upon the particular product. *Figure 6* illustrates one of many possible downstream processing configurations. The first stage is primarily for clarification and concentration. In bacterial fermentation, the product remains within the cell. The first step is to separate the cells from the cell debris and the broth. This can be performed using a high-speed centrifuge, micro-filtration membrane or a filter press. Protein purification schemes include cell disruption, followed by initial fractionation, then a secondary fractionation, and finally a polishing step.

The next stage is to mix the clarified material with a neutralizing buffer. The target protein must then be separated from other proteins and any other components that remain in the clarified mix (lipids,

nucleic acids, carbohydrates, etc.) One technology that is commonly used for the purification step is ion-exchange chromatography. The equipment includes columns that are packed with beads that absorb the protein molecules as they are pumped through. The captured proteins are then desorbed by adding competing ions (salt) and the proteins are eluted from the column in the order determined by the strength of the bonds. The bond strength is in turn determined by the charges on the individual amino acids. When the target molecules elute from the column, they can be diverted to the pure product stream which then passes through a gel permeation system to remove the salt. The last procedure in the bulk manufacturing facility is to freeze dry the purified API which should now be at least 99.99% pure.

PAT in Large Molecule Bulk Pharma

There is the opportunity to make use of online process analytical technology throughout downstream processing. The two areas getting the most attention, however, appear at either end of this flow diagram. Monitoring the fermentation process online is important because of the possibility of significant batch-to-batch variation. A similar case can be made for the freeze drying (Lyophilization) or vacuum drying processes. The remainder of this applications note will concentrate on the optimization of the fermentation process.

Process Optimization

The primary objective of fermentation process development is to ensure that the sterile cultivation medium contains all the necessary nutrients at concentrations that will first promote biomass growth and then force rigorous production of the target molecule. Microbes require sources of carbon, nitrogen, oxygen and inorganic ions as well as some species-specific nutrients. The numerous growth medium candidates are best compared by using online analysis of the effluent gas. Discrete analyzers can be used for measuring oxygen and carbon dioxide but a more comprehensive and precise method of monitoring metabolic activity is to use a multistream process mass spectrometer. The most popular model for this application is the Thermo Scientific Prima δB illustrated in *figure 7*. A single analyzer is typically used to monitor a large number of heated benchtop fermentors (although fermentation is exothermic, small scale fermentors tend to lose more heat than they produce). The software configurable nature of mass spectrometry allows the microbiologist to look for small concentrations of molecules that might indicate problems with a selected batch that isn't performing as expected. For example, ethanol can be produced if there is too much carbohydrate available. Another important control parameter in the fermentation process is the respiratory quotient (RQ). This is the ratio of the carbon dioxide evolution rate (CER) and the oxygen uptake rate (OUR). This ratio is used for the online derivation of biomass and as an indicator of the substrates being consumed and the products being formed. In addition to the permanent gases, the aqueous concentrations of many organic species can be determined by analysis of the vent gas.

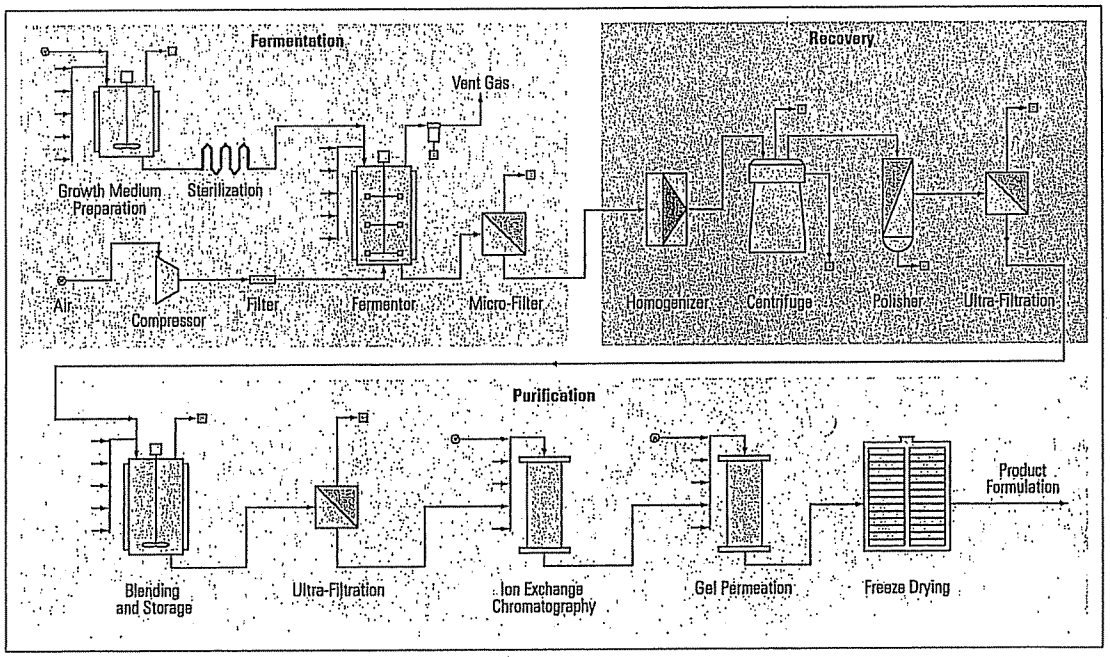

figure 6 – Downstream process schematic

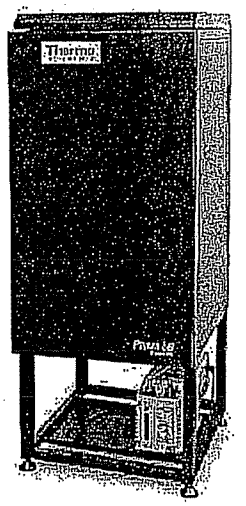

figure 7 — The Thermo Scientific Prima δB

Multistream Sample Manifolds

When engineering a system to monitor the sparge and vent gases of a number of fermentors, care must be taken to provide a sample stream selection device that is both fast and reliable. The Thermo Scientific Rapid Multistream Sampler (RMS) is a zero dead-volume system that has over 5,000 operational years of service. The vent gas from each vessel continuously flows through the device to a common vented chamber with one stream at a time being diverted by the rotating sample head into the double bypass capillary. The design provides a heated, inert path for the selected sample stream without allowing the possibility of cross-port contamination. The location of the selector is monitored by a dedicated processor that also controls three temperature zones and monitors a flow alarm that provides operator warning in the event that a selected stream has a low sample flow. The RMS comes in 32 and 64 port variants that can be installed two at a time on a single analyzer thereby providing a maximum capacity of 127 ports (port 1 of the primary RMS is reserved for connection to the secondary RMS output).

Mass spectrometers operate by ionizing neutral molecules or atoms and they separate these components according to the mass to charge ratio. The ionization is carried out by bombarding the gas sample with an electron beam from a hot filament. The technique chosen to separate the ions in the Prima δB system is the variable magnetic sector.

In the magnetic sector analyzer, the trajectory of the ions is controlled by a variable magnetic field allowing ions of interest to be sequentially collected onto a single detector. Thus, the mass spectrometer is able to scan the sample gas to identify both known and unknown species. The advantage of this type of analyzer is that it produces flat-topped peaks where the intensity of the peak is proportional to the concentration of the gas. This optimum peak profile provides the most stable and precise method for measuring the gas concentration and significantly improves specificity when there are overlaps in the mass spectra of several gases. The Prima δB mass spectrometer also benefits from a variable ion energy supply that allows the mass range to be adjusted to match the species that are selected for analysis. The benefit from this is that the width of the peak is maximized; an attribute that guards against drift effects.

The measured signal is processed by modern, surface-mount electronics and the calculated concentrations are stored in battery-backed 'flash' memory that can be accessed directly using a variety of industry standard protocols. The embedded computer uses an industrial ARM processor that runs a multi-threaded, real-time operating system that is capable of running the system in stand-alone mode. This type of architecture is important for mission-critical installations and is designed to support 21CFR Part 11 compliance.

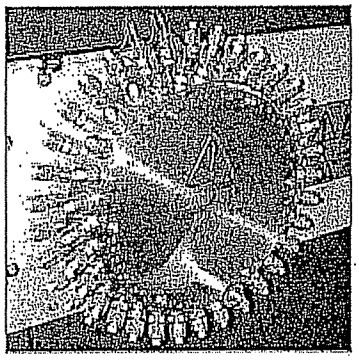

figure 8 — Rapid Multistream Sampler (RMS)

Analysis Speed versus Precision

Figure 11 illustrates the trade-off between analytical precision and speed of analysis. Depending upon the number of fermentors being monitored and the duration of each batch, the analysis of individual streams

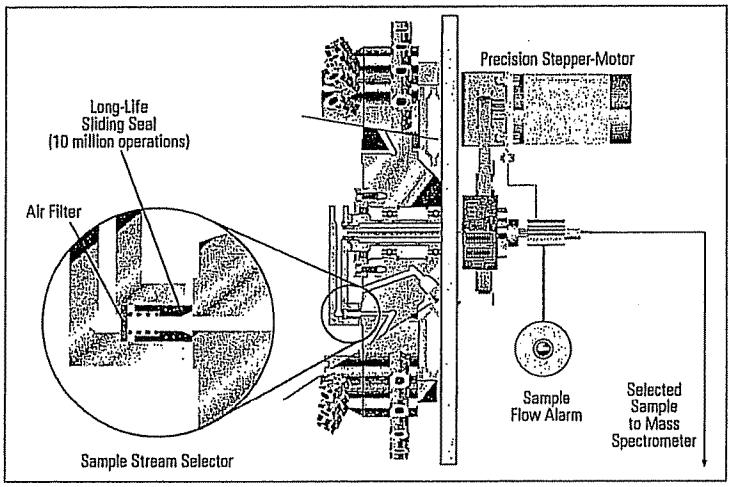

figure 9 – RMS cut-away

can be adjusted to optimize the measurement schedule. The top trace shows the oxygen data points measured with the analysis time set to 30 seconds. The standard deviation (SD) of these data, over 24 hours, is 13.4 ppm (<0.01% relative). The lower trace shows the oxygen data measured at the same point but with the analysis time at three seconds. The SD deteriorates to 27 ppm (0.013%).

The flexibility of the mass spectrometer in terms of analytical methods, speeds and sequencing ensures that a single analyzer can provide a wealth of valuable information during biological process development and scale-up. In addition to the precise determination of RQ, the Prima δB provides for the measurement and computation of a number of other important parameters:

- Verify absence of contamination pre-inoculation
- Periodically carry out a full mass scan to 'fingerprint' normal and abnormal fermentations
- Produce complex outputs suitable for training neural networks
- Allow for the accurate online computation of biomass, growth kinetics and potency
- Identify new molecular species and improve process intimacy.

Since the mass spectrometer measures all the gas concentrations in this equation, it is able to calculate a precise number for RQ. It should be noted that the possible errors that are introduced when accurate flow correction is not carried out can be quite significant. It should also be noted that when RQ measurements are calculated based on flow rates and batch volumes, errors are introduced by imprecise measurement. Typical mass flow controllers provide a flow measurement accurate to only ±1 to 5% and batch volume is difficult to measure online.

In fed-batch fermentation, the initial nutrient levels are limited to ensure that the cells don't get overloaded (oxidative capacity of the cells exceeded) because this condition can result in contamination that inhibits cell growth. When there are indications that the substrate is becoming depleted, the nutrient is added in several doses. This control strategy increases the cell density and

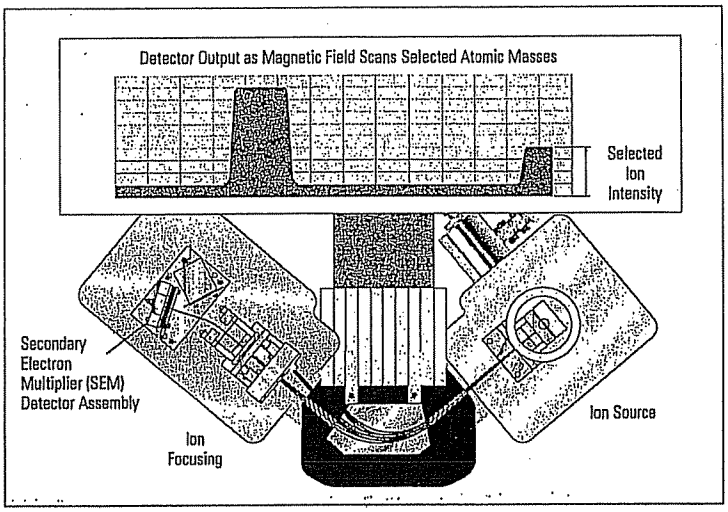

figure 10 – Scanning magnetic sector mass spectrometer

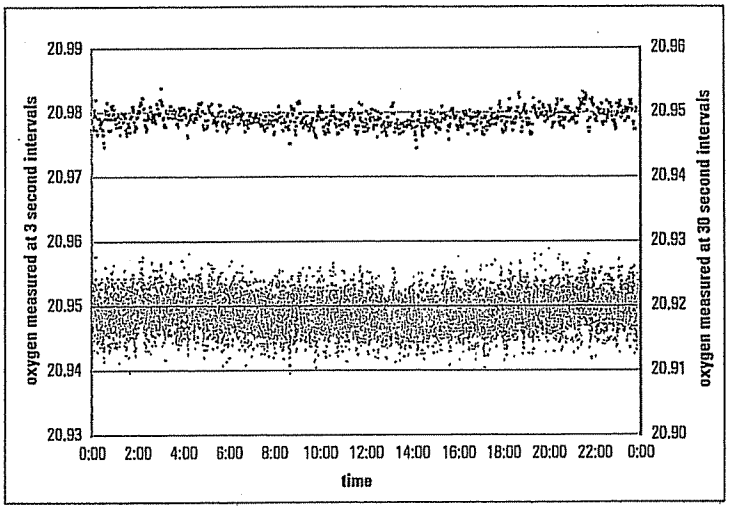

figure 11 – Oxygen precision for 3 second and 30 second analysis times

How RQ is Calculated

CO_2 evolution rate = %volume of $CO_{2_{out}}$ x $flow_{out}$ - %volume $CO_{2_{in}}$ x $flow_{in}$

O_2 uptake rate = %volume of $O_{2_{in}}$ x $flow_{in}$ - %volume $O_{2_{out}}$ x $flow_{out}$

$$RQ = \frac{(\%\text{volume of } CO_{2_{out}} \times flow_{out} - \%\text{volume } CO_{2_{in}} \times flow_{in})}{(\%\text{volume of } O_{2_{in}} \times flow_{in} - \%\text{volume } O_{2_{out}} \times flow_{out})}$$

%volume of $N_{2_{in}}$ x $flow_{in}$ = %volume of $N_{2_{out}}$ x $flow_{out}$
(except for rare N_2 fixing micro-organisms)

This leads to the equation:

$$RQ = \frac{\{\%\text{vol of } CO_{2_{out}} \times (\%\text{vol of } N_{2_{in}} / \%\text{vol of } N_{2_{out}}) - \%\text{vol of } CO_{2_{in}}\}}{\{\%\text{vol of } O_{2_{in}} \times - \%\text{vol of } O_{2_{in}} \times (\%\text{vol of } N_{2_{in}} / \%\text{vol of } N_{2_{out}})\}}$$

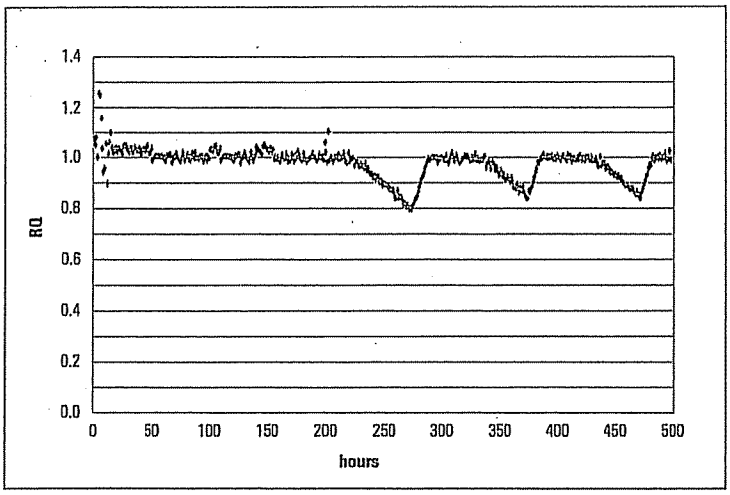

figure 12 – The use of RQ measurement to control E.coli fed-batch fermentation

potency over conventional batch fermentation processes. In some situations, it is difficult to determine the dosing interval online and the process development engineer has to rely on Multivariate Statistical Process Control (MSPC) which might include a combination of Principal Component Analysis (PCA), Partial Least Squares (PLS) and Kernel Density Estimation (KDE). These mathematical models can take significant time to develop, and they take their inputs from a number of probes and transducers that can suffer from drift. In some situations, a simple control scheme can be developed that uses the RQ value, produced by the mass spectrometer, to control nutrient additions. *Figure 12* shows a typical RQ trend in a fed-batch process utilizing E.coli for protein production.

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